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Effects of a single intratracheal administration of phenanthraquinone on murine lung

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ABSTRACT: **Although several studies have reported that diesel exhaust particles (DEP) affect cardiorespiratory health in animals and humans, the responsible components in DEP for the effects remain to be defined. Diesel exhaust particles contain quinones that can catalyse the generation of reactive oxygen species, resulting in the induction of oxidative stress. Oxidative stress can correlate with a variety of diseases and health effects. In the present study, we investigated the effects of phenanthraquinone — a relatively abundant quinone in DEP — on lung inflammation and the local expression of cytokine proteins in mice as a measure of oxidative damage. The animals were randomized into two experimental groups that received vehicle or phenanthraquinone by intratracheal instillation. The cellular profiles of bronchoalveolar lavage fluid (BALF) and local expression of cytokines were evaluated 24 and 48 h after the instillation. Phenanthraquinone challenge revealed an increase in the numbers of neutrophils and eosinophils in BALF as compared to vehicle challenge ($P < 0.05$ at 48 h post-instillation). Phenanthraquinone induced the lung expression of interleukin (IL)-5 and eotaxin 48 h and 24 h after the challenge, respectively. These results indicate that intratracheal exposure to phenanthraquinone induces recruitment of inflammatory cells, at least partly, through the local expression of IL-5 and eotaxin. Copyright © 2005 John Wiley & Sons, Ltd.**

KEY WORDS: phenanthraquinone; diesel exhaust particles; eosinophil; cytokine

Introduction

Diesel exhaust particles (DEP) are major contributors to the atmospheric particulate air pollution in metropolitan areas. They have been correlated with lung cancer, pulmonary fibrosis, chronic alveolitis (McClellan, 1987), oedematous changes (Ichinose *et al*., 1995), and airway inflammation with hyperresponsiveness (Sagai *et al*., 1996). Previously, we have reported the enhancing effects of DEP on allergic asthma in murine models (Takano *et al*., 1997, 1998) . We have shown also that DEP enhance neutrophilic lung inflammation related to endotoxin (Takano *et al*., 2002).

Diesel exhaust particles consist of carbonaceous nuclei and a vast number of organic compounds such as polyaromatic hydrocarbons, aliphatic hydrocarbons, heterocycles and quinones. Recently, we have shown that intratracheal instillation with residual carbonaceous nuclei of DEP after extraction with dichloromethane rather than the organic chemicals in DEP enhances the neutrophilic lung inflammation related to endotoxin (Yanagisawa *et al*., 2003). On the other hand, previous *in vitro* studies have indicated that exposure of macrophages to organic chemicals extracted from DEP results in induction of apoptosis (Hiura *et al*., 1999) and increases gene expression of the oxidative stress-inducible protein haeme oxygenase-1 (Li *et al*., 2000). Organic chemicals in DEP also can affect inflammatory effector cells, including neutrophils, eosinophils and macrophages (Terada *et al*., 1997; Yang *et al*., 1997; Kanemitsu *et al*., 1998), and trigger the release of proinflammatory molecules such as interleukin (IL)-1, IL-8 and tumour necrosis factor (TNF)-α, which are regulated on activation and normal T cells are expressed and secreted *in vitro* (Yang *et al*., 1997; Fahy *et al*., 1999). However, the components of DEP responsible for its effects on the respiratory system remain to be defined, especially *in vivo*.

Previously, a variety of quinones have been identified as DEP components (Schuetzle *et al*., 1981; Schuetzle, 1983; Cho *et al*., 2004). Quinones have toxicological properties to serve as alkylating agents and to interact with, for example, flavoproteins to generate reactive oxygen species (ROS), which can induce biological

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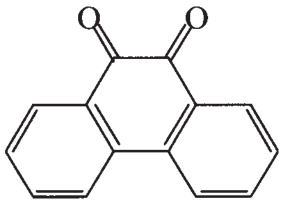

Figure 1. Structure of phenanthraquinone

injury (O'Brien, 1991; Monks *et al*., 1992; Bolton *et al*., 2000). Among them, phenanthraquinone (Fig. 1) is recognized as a relatively abundant quinone in DEP (Schuetzle, 1983). Phenanthraquinone has a potential to act as a redox cycling quinone, generating thiol oxidants such as hydrogen peroxide. Indeed, we have demonstrated the role of phenanthraquinone in oxidative stress *in vitro* (Kumagai *et al*., 2002). On the other hand, our laboratory has demonstrated previously that DEP can induce lung injury through the enhanced formation of oxidative stress (Lim *et al*., 1998; Sanbongi *et al*., 2003), suggesting that phenanthraquinone may induce lung injury.

In the present study, we explored the effects of a single intratracheal administration of phenanthraquinone on the respiratory system *in vivo*. We also determined the effects of phenanthraquinone on the lung expression of cytokines and chemokines.

Materials and Methods

Animals and Study Protocol

The studies reported were carried out in accordance with the Declaration of Helsinki and with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the US National Institutes of Health. All animal studies were approved by the Institutional Review Board. Male ICR mice (6 weeks old, 29–33 g) were purchased from Japan Clea Co. (Tokyo, Japan) and fed a commercial diet (Japan Clea Co.) and water *ad libitum*. The mice were housed in an animal facility that was maintained at 24–26 °C with 55–75% humidity and a 14/10 h light/dark cycle as described previously (Takano *et al*., 1997). Mice were treated with vehicle or phenanthraquinone (Sigma Chemical Co., St Louis, MO). The vehicle group received intratracheally 100 μl of phosphate-buffered saline (PBS) at pH 7.4 (GIBCO BRL, Life Technology, Grand Island, NY) containing 0.05% Tween 80 (Nacalai Tesque, Kyoto, Japan) and 4% dimethyl sulphoxide (DMSO). The phenanthraquinone group received 1 μg per mouse of phenanthraquinone dissolved in 100 μl of the same vehicle. Intratracheal inoculation was conducted using a polyethylene tube under anaesthesia with 4% halothane (Hoechst Japan, Tokyo, Japan), as described previously (Takano *et al*., 1997, 2000). All mice were killed by deep anaesthesia using diethyl ether 24 or 48 h after the instillation.

Bronchoalveolar Lavage

The trachea was cannulated after exsanguination. The lungs were lavaged with 1.2 ml of sterile saline at 37 °C, instilled bilaterally by syringe. The bronchoalveolar lavaged fluid (BALF) was harvested by gentle aspiration. This procedure was conducted two more times. The average volume retrieved was 90% of the 3.6 ml that was instilled; the amounts did not differ by treatment. The fluid collections were combined and cooled to 4 °C. The BALF was centrifuged at 1200 *g* for 10 min and the total cell count was determined on a fresh fluid specimen using a haemocytometer. Differential cell counts were assessed on cytological preparations. Slides were prepared using a Cytospin (Tomy Seiko, Tokyo, Japan) and were stained with Diff-Quik (International Reagents Co., Kobe, Japan). A total of 500 cells were counted under oil immersion microscopy ($n = 5$ in each group).

Quantitation of Cytokines in Lung tissue Supernatants

In a separate series of experiments, animals were exsanguinated and the lungs were subsequently homogenized with 10 mM potassium phosphate buffer (pH 7.4) containing 0.1 mM ethylenediaminetetraacetic acid (Sigma Chemical Co.), 0.1 mM phenylmethanesulphonyl fluoride (Nacalai Tesque), 1 μM pepstatin A (Peptide Institute, Osaka, Japan) and 2 μM leupeptin (Peptide Institute), as described previously (Takano *et al*., 2002). The homogenates were then centrifuged at 105 000 *g* for 1 h. The supernatants were stored at −80 °C. Enzyme-linked immunosorbent assays (ELISA) for IL-4 (Endogen, Cambridge, MA), IL-5 (R&D Systems, Minneapolis, MN) and eotaxin (R&D Systems) in the lung tissue supernatants were conducted using matching antibody pairs according to the manufacturer's instruction ($n = 5$ in each group). The second antibodies were conjugated to horseradish peroxidase. Subtractive readings of 550 nm from the readings at 450 nm were converted to pg ml^{-1} using values obtained from standard curves generated with limits of detection of 3, 1.5, and 2 pg ml^{-1}, respectively.

Statistical Analysis

Data were reported as the mean ± SEM. Differences among groups were determined using analysis of variance (Stat View, version 4.0; Abacus Concepts, Inc., Berkeley, CA) as described previously (Takano *et al*., 1997).

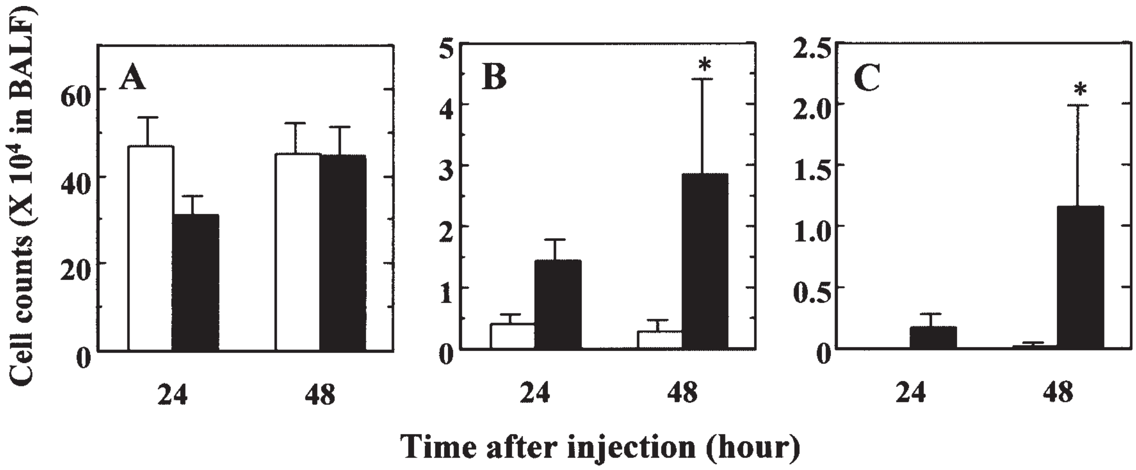

Figure 2. Alterations in the numbers of macrophages (A), neutrophils (B) and eosinophils (C) in bronchoalveolar lavage fluid (BALF) following a single intratracheal administration of phenanthraquinone (1 μg per mouse) in mice. Twenty-four hours and fourty-eight hours after the intratracheal administration of vehicle or phenanthraquinone, lungs were lavaged for the analysis of BALF. Differential cell counts were assessed on cytological preparations stained with Diff-Quik. White bar: vehicle treatment. Black bar: phenanthraquinone treatment. Results are means ± SEM (n = 5); * $P < 0.05$ versus vehicle-treated mice

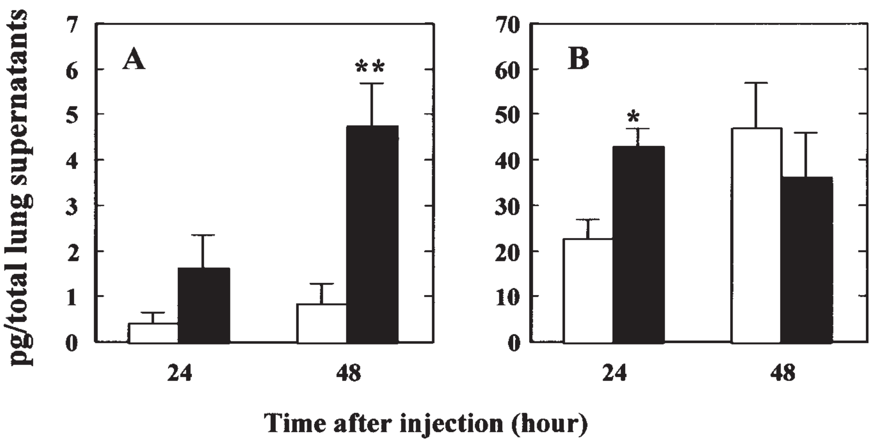

Figure 3. Protein levels of interleukin IL-5 (A) and eotaxin (B) in the lung tissue supernatants after challenge with phenanthraquinone. Lungs from mice were obtained 24 and 48 h after the administration of vehicle or phenanthraquinone. The IL-5 (A) and eotaxin (B) levels in the lung tissue supernatants were measured by enzyme-linked immunosorbent assays. White bar: vehicle treatment. Black bar: phenanthraquinone treatment. Results are means ± SEM (n = 5); * $P < 0.05$ versus vehicle-treated mice; ** $P < 0.01$ versus vehicle-treated mice

Results

To determine the effects of phenanthraquinone on the respiratory systems, we investigated the cellular profile of BALF 24 h and 48 h after the intratracheal instillation with vehicle or phenanthraquinone (1 μg per mouse). As shown in Fig. 2A, phenanthraquinone did not change the number of macrophages in BALF at either 24 h or 48 h after the intratracheal challenge. Phenanthraquinone also did not induce a significant increase in the numbers of neutrophils and eosinophils 24 h after the challenge (Figs. 2B and 2C). However, phenanthraquinone caused an increase in the numbers of neutrophils and eosinophils in BALF 48 h after the challenge as compared to vehicle (Figs. 2B and C).

To investigate the effects of phenanthraquinone on the lung expression of proinflammatory cytokines and chemokines, we compared the protein levels of IL-4, IL-5 and eotaxin in the lung tissue supernatants among the experimental groups 24 and 48 h after the intratracheal instillation. Treatment with phenanthraquinone significantly elevated the expression of IL-5 at 48 h ($P < 0.01$ vs vehicle treatment: Fig. 3A) and eotaxin at 24 h ($P < 0.05$ vs vehicle treatment: Fig. 3B). On the other hand, the lung expression of IL-4 did not show a significant change between the vehicle and phenanthraquinone groups (data not shown).

Discussion

The present study has shown that a single intratracheal exposure to phenanthraquinone causes the recruitment of inflammatory cells such as neutrophils and eosinophils to the murine airways. Phenanthraquinone enhances the lung expression of IL-5 and eotaxin.

We have demonstrated previously that DEP can cause respiratory effects, including oedematous changes (Ichinose *et al*., 1995), carcinogenesis (Ichinose *et al*., 1997), airway inflammation with hyperresponsiveness (Sagai *et al*., 1996) and enhancement of both allergic lung inflammation (Takano *et al*., 1997, 1998) and neutrophilic lung inflammation (Takano *et al*., 2002) *in vivo*. However, responsible components of DEP for the enhancement remain to be determined, especially *in vivo*.

Quinones are considered to be toxicologically important components of air pollution. They have been found in ambient particulate matter (Fraser *et al*., 2003; Cho *et al*., 2004), automotive exhaust emissions (Schuetzle *et al*., 1981) and wood smoke particles (Fine *et al*., 2001). Quinones have potent nephrotoxicities, neurotoxicities and carcinogenicities (Monks and Lau, 1992), and cause mitochondrial dysfunction (Henry and Wallace, 1996). Some quinones can generate ROS, including superoxide, hydrogen peroxide and ultimately hydroxyl radical, resulting in cellular damage (Bolton *et al*., 2000). Phenanthraquinone is one of the quinones found at significant concentrations in DEP (Schuetzle, 1983; Cho *et al*., 2004). Furthermore, we have shown previously that phenanthraquinone can induce the oxidation of sulfhydryls via the production of ROS (Kumagai *et al*., 2002). Based on these previous studies, we hypothesized that phenanthraquinone may play a role in the toxicities of DEP on respiratory systems. In the present study, we first demonstrated that the single intratracheal instillation with phenanthraquinone induced the recruitment of inflammatory cells such as neutrophils and eosinophils to the murine airways.

The mechanisms by which inflammatory cells are mobilized to the airways are not presently defined. The recruitment of eosinophils to the inflammatory sites is a complex process that is regulated by a number of cytokines such as IL-1β, IL-4, IL-5, IL-12, granulocyte macrophage colony-stimulating factor and TNF-α, and chemokines that include eotaxin (Broide and Sriramarao, 2001; Shakoory *et al*., 2004; Wong *et al*., 2004). Among them, eotaxin and IL-5 appear to be more important for the development of eosinophil recruitment. IL-5 mobilizes eosinophils from the bone marrow (Broide and Sriramarao, 2001) and functions with eotaxin to regulate eosinophil homing and migration to sites of inflammation (Collins *et al*., 1995). In the present study, intratracheal administration of phenanthraquinone enhanced the lung expression of IL-5 and eotaxin compared to that of vehicle. These results indicate that the recruitment of eosinophils to the murine airways induced by phenanthraquinone may be mediated, at least partly, via the lung expression of eotaxin and IL-5. We have reported previously that DEP enhance lung expression of proinflammatory cytokines and chemokines related to antigen and bacterial endotoxin. Also, we have demonstrated that DEP-induced lung inflammation is concomitant with the increase in nitric oxide syntheses and the decrease in superoxide scavenger (Lim *et al*., 1998). Furthermore, we have demonstrated recently that DEP-induced lung inflammation is in parallel with the formation of nitrotyrosine and 8-OHdG, which are proper oxidative stress markers (Sanbongi *et al*., 2003). Therefore, it may be hypothesized that phenanthraquinoneinduced lung inflammation may be mediated through the enhancement of oxidative stress. Further studies are needed to clarify the hypothesis.

In conclusion, we have shown that the single intratracheal exposure to phenanthraquinone causes the recruitment of inflammatory cells such as neutrophils and eosinophils to the murine airways and enhances the lung expression of IL-5 and eotaxin. Our results indicate that exposure to phenanthraquinone may elicit lung inflammation and may play a role, at least partly, in the pathogenesis of pulmonary toxicities of DEP *in vivo*.

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